

REAL TIME REFERENCE CONTROLLED REVERSE DIFFUSION QUANTITATION OF MICROLEAKAGE: STANDARDIZATION OF THE METHODS*

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ان مراجعة البحوث والمقالات العلمية تبين الحاجة إلى وجود طريقة دقيقة لقياس كمية السوائل المتسربة حول الحشوات السنية .
كما يستنتج من هذه البحوث ان هناك طريقة يمكن استخدامها في المعامل لقياس كمية التسرب من خلال فترات زمنية محددة دون احداث ضرر للأسنان .
وحيث ان هناك عدة عوامل خارجية تؤثر على هذه الطريقة لذلك وجب أخذ الحيطة والدقة لوضع طرق ثابتة وغير متغيرة لقياسها .
هذه الدراسة تصف نتائج عشرة تجارب استخدمها الباحثون في هذا البحث من أجل اختيار أفضلها فيما يخص المواد والطرق المستخدمة بها .
كما تبين هذه الدراسة جميع النتائج المتحصلة من هذه التجارب .
وبناء على هذه النتائج الأولية فقد تم اختيار الطريقة التي اعتقد الباحثون أنها دقيقة لتقدير كمية التسرب الدقيق للسوائل كما تم تحديد المعايير الثابتة لقياسها حول عدد من المواد والطرق بالنسبة للحشوات الموضوعية داخل السن أو التي تغطيه من الخارج أو المستخدمة في حشو أفنية الجذور ونتائج هذه الدراسات سوف تنشر لاحقاً .

An intensive literature review on evaluating microleakage suggested that a quantitative method is needed. Further, the review concluded that an *in vitro* reference controlled reverse diffusion method is one which is likely to be non- destructive, simple, objective and capable of quantitating microleakage in real time and over extended durations. Since the proposed method can be influenced by extraneous variables, careful standardization of the experimental procedure was necessary. Ten experiments leading to the choice of the proper material/method of testing were conducted. The results of all experiments are reported. Based on the results of these pilot studies, a well calibrated overall experimental procedure for quantitating microleakage *in vitro* was determined. The standardized reference controlled procedure is recommended for quantitating microleakage of various materials and techniques utilized in intra-, extra-, and endodontic restorations. Reports of results of the latter studies are in preparation.

Received 07/1/95; revised 01/10/95; accepted 03/10/95.

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t This study was supported by King Abdulaziz City for Science and Technology. Research Grant No. AT-12-57, P.O. Box 6086, Riyadh 11442, Saudi Arabia.

Introduction

The need for a method capable of quantitating microleakage was recently justified.¹ Further, an *in vitro* method capable of such quantitation was proposed. The method, as its name implies, relies upon reverse diffusion and quantitates microleakage in real time (at any instant of leakage and immediately) relative to a control. Briefly, the

method consists of depositing a known count of radioactive material on the floor of a cavity. The cavity is restored, immersed in a non-radioactive medium and left to leak. Aliquot samples are taken from the immersion medium at various time intervals. The radioactivity in each sample is determined and compared to that leaked from a group of open cavities at the same time interval. The amount of microleakage that occurred from the cavity (open or restored) at each time interval can be computed from the radioactivity detected in each aliquot sample at the corresponding time interval. The procedure is continued until no further leakage occurs or until microleakage becomes 100%.¹

Yielding reliable data requires choice of optimal materials and methods for the overall experimental protocol. The proposed experimental method of Real Time Reference Controlled Reverse Diffusion is new as well as sensitive. Since true quantitation of microleakage is sought, all variables that may affect the accuracy of results should be tested. Accordingly, the chief principles for experimental standardization; precision and accuracy, are implemented following the general principles for statistical analysis methodology.²

The experimental method consists of depositing a radioisotope on the floor of a cavity. The most suitable radioisotope must be experimentally selected. The radioisotope is placed in the cavity together with its liquid carrier. The carrier must be dried. A consistent and efficient method of drying must be determined. The radiotracer may seep through the dentinal tubules. Absence of such seepage needs to be experimentally assured. The given volume of VA-Oralube³ artificial saliva in which such a tooth/root is immersed needs to be correctly selected. The immersed sample is stored at 37°C with gentle shaking. Temperature and shaking may cause substantial evaporation of aliquot and cause an increase in its radioactivity level. The validity of such hypothesis must be tested. Periodically an aliquot sample is taken from the soaking medium, the proper volume for the sample needs to be determined. The aliquot sample is mixed with a scintillation cocktail. The cocktail least capable of masking the radioactivity needs to be experimentally identified.

The goal of this study was to conduct ten experiments in an effort to standardize the overall testing method and assure its reliability.

Materials and Methods

Choice of a suitable scintillation cocktail with high radioactivity recovery.

In the experimental procedure, a radioactive material leaks out of the cavity into saliva (reverse diffusion). A sample of the saliva containing the leaked radioisotope is taken and mixed with a scintillation cocktail in preparation for reading the number of radioactivity counts using a scintillation counter. The essential considerations in the choice of scintillation solutions⁴⁶ were strictly followed in order to attain the optimum counting efficiency, sensitivity, and accuracy. Some scintillation cocktails mask certain amount of radioactivity. The more the masking effect, the less efficient is the cocktail. The less the masking effect, the more efficient is the cocktail or the better is the recovery (of radioactivity). An ideal scintillation cocktail is one that yields 100% recovery, which of course does not exist. Two of the most commonly used cocktails are Optiphase* and Lumagel*. A determination as to which of these two cocktails is most efficient in recovering the radioactivity is needed.⁷

Since the amount of radioactivity in the cocktail also influences recovery, six levels of radioactivity (10, 20, 30, 40, 50 and 60 μCi of the radiotracer) were used. The amount of recovery is also influenced by whether the cocktail is alone or mixed with saliva. Each level of radioactivity was tested with each of the two cocktails in one of two conditions; when the cocktail (10 μCi) was alone or when it was mixed with artificial saliva (10 mL). Thus, the experiment consisted of 6 levels of radioactivity, two types of cocktail and two conditions of each cocktail (pure or mixed with saliva). Number of experimental conditions was $6 \times 2 \times 2 = 24$. Each experimental condition was repeated six times to obtain the mean. Thus, 144 specimens were examined. Having established the recovery efficiency of each cocktail in its pure form, a correlation coefficient R was determined to see how the cocktail would perform when mixed with saliva relative to its performance alone. The radioisotope used was ³H-Alanin (47 curies/mmol). The activity was measured in a Liquid Scintillation Counter. §

* FSA Laboratory Supplies, England.

§ Lumac, The Netherlands.

† Radio Chemical Center, Amersham, England.

§ Model 1215 LKB Wallace Rackbeta, Finland.

Study of saliva and aliquot volumes

The volume of artificial saliva to be used as a medium for the leaking sample may play a role in accuracy of results. After establishing the volume of immersion saliva, the appropriate volume withdrawn every time a radioactivity count is to be determined needs also to be established. The withdrawn saliva will contain radioactivity and is called aliquot.

Using 20 conical flasks, five samples of each of four different volumes of artificial saliva (10 mL, 20 mL, 50 mL and 100 mL) were placed. A dose of 10 μL (2.5×10^5 counts per minute) of ^3H -Alanine was added to each volume of artificial saliva. Aliquots of 10 μL , 50 μL and 100 μL were withdrawn from each saliva volume at various time intervals. The mean radioactivity in each dose from five flasks was determined. A one-way multiple analysis of variance microcomputer program was adopted for the statistical analysis of these data.⁸

Volume change of artificial saliva due to water bath temperature

In an attempt to represent prevailing oral conditions, it was decided to conduct the microleakage experiments at mouth temperature (37°C) with gentle shaking (80 times/minute). The influence of 37°C on evaporation of the medium, hence on concentration of the aliquot, was of concern. The objective of this study was to experimentally determine whether testing at 37°C with shaking and periodic unstoppering of the flask containing the tooth and aliquot (to take a sample) will cause loss in saliva volume and increased radioactivity concentration in the aliquot.

In each of ten conical flasks, 20 mL artificial saliva and 10 μL ^3H -Alanine (2.5×10^5 counts per minute) were added. Five of the flasks were stoppered, placed in the water-bath and kept at 37°C with gentle shaking (80 times/minute). The other five flasks were stoppered and kept at room temperature (25°C). At times representing the experimental procedure, and up to 47 days, aliquots of 50 μL were collected from each of the ten flasks and placed in the scintillation vials containing 10 mL Optiphase. At each time interval when 50 μL of aliquot was removed, 50 μL artificial saliva was added to the flasks to replace the withdrawn aliquot. Radioactivity in each specimen was measured using the liquid scintillation counter.

Efficacy of nail enamel as a sealant

In order to determine that the leakage occurs only at the restoration-cavity wall interface, the remaining portions of the tooth must be sealed to prevent any potential exchange of radioactivity with the medium. It is well known that the enamel surface of teeth, both in the mouth and after extraction contains microscopic fractures. It is also known that the cementum around the roots of the teeth contains cracks, principal and accessory root canals. Therefore, it is necessary to isolate all surfaces, other than the portion of the surface where the restoration is made, from the medium where the restored tooth is soaked. This "walling off" is achieved by coating the non-restored surfaces with nail enamel up to the cavo-surface margin. Hence, tests were conducted to ascertain the sealing ability of nail enamel in blocking microscopic gaps before this material was chosen to coat the teeth. Extracted human molars which had been restored with dental amalgam with the radioactive tracer at the bottom of the cavities were used for the test. Four specimens were completely coated with nail enamel exposing only the restorations. Four other specimens had two layers of nail enamel all around the teeth including the restorations. Each of the four teeth in each group was immersed in artificial saliva and exposed to shaking for one week. Samples were taken from each of the eight aliquots. Radioactivity presence in each sample was tested using the liquid scintillation counter.

Retention of radioactivity by nail enamel

In order that the radioactivity collected from the aliquot at various intervals represents what has actually leaked from the cavity, it is important to ascertain that nail enamel on the tooth surface does not absorb and retain some of the released radioactivity.

In each of 5 conical flasks, 20 mL of artificial saliva and 10 μL of ^3H -Alanine (2.5×10^6 counts per minute) were added. In each flask an upper molar tooth completely sealed with nail enamel was placed. Flasks were stoppered and placed in the water-bath and kept at 37°C with gentle shaking (80 times/minutes).

At time intervals representing the experimental procedure, and up to 47 days, aliquot of 50 μL was collected from each flask and placed in a scintillation vial containing 10 mL Optiphase. Radioactiv-

ity in each vial was measured using the liquid scintillation counter.

Potential seepage of the radioactive tracer into the dentinal tubules

As has been described above, in the reverse diffusion method^{9,12} a known quantity of the radioactive tracer is placed at the bottom of the cavity before it is restored. It is assumed that this radioactive tracer will remain on the floor of the cavity to leak only through the restoration-cavity wall interface. It has previously been established that the radioactive material is partially bound to the floor of the cavity and is not available immediately to leak.¹ However, since the radioactive material is deposited on the cavity floor which is made up of dentinal tubules, one may assume that some of the radioactivity may leak through the dentinal tubules to certain depths within dentin or even into the pulp chamber, thus becoming completely unavailable for leakage. Thus, it was necessary to investigate whether such pulpward seepage does occur.

Class V cavities were prepared in five human molars, the whole tooth surface (except the cavity) was coated with two layers of nail enamel and 5 μ L of ³H-Alanine was placed in each cavity. The radioactivity carrier was dried by a visible light source and the cavities were restored with amalgam. The teeth were placed in artificial saliva at 37°C and gentle shaking (80 times/minute) for three weeks. Each tooth was sliced parallel to its long axis and to the floor of the cavity. Each slice was approximately 1 mm thick. The two slices obtained from between the pulp and the floor of the cavity were analyzed for any radioactivity. Each section was immersed in 20 mL of saliva at 37°C and gentle shaking for a week. Samples from the aliquot were counted for any radioactivity. A count, more than that of the background count would indicate the presence of radioactive tracer in the dentinal tubules and hence, the presence of intratubular seepage.

Selection of appropriate radiotracer

The type of radiotracer to be used in quantitating microleakage is important. The nature of the radiotracer and its molecular size should be carefully selected. The use of calcium radiotracer, for example, is not advisable due to its affinity to tooth structure. In fact, the use of any electrolytic radiotracer

will not be favorable since its size is too small to represent the size of molecules leaking around a dental restoration. Amino-acid radiotracers, on the other hand, are macroorganic molecules and of a size close to that of bacterial toxin molecular size. Accordingly, using amino-acid radiotracers in microleakage evaluation may render such studies clinically relevant.⁹ Among the amino-acid radiotracers, ³H-labelled amino-acids and ¹⁴C-labelled amino-acids are most useful. In this research, ³H-labelled amino-acids were preferred to ¹⁴C-labelled compounds since the former have high specific activities (47 Curies/mmol) making them more reliable in such radioisotope biological tests.

There are several ³H-labelled amino-acids. Among the most useful are ³H-Lucine, ³H-Proline and ³H-Alanine. The test evaluates the influence of the tooth/restoration surface features on the microleakage of the radiotracer. The physico-chemical properties of the radiotracer itself, mainly its water solubility and partition coefficient between the intra and extra tooth fluids may also influence the extent as well as the rate of its release, hence, the collected data. In order to investigate this postulation, the three ³H-labelled amino acids were compared to identify the radiotracer with the highest release.

Class V cavities (2x2x5 mm) were prepared in 18 human molar teeth. All teeth were coated with two layers of nail enamel up to the cavo-surface margins. The teeth were divided to 3 groups of 6 teeth each. Each group received one of the radiolabeled ³H-Amino acids. In each of the 6 cavities of each group, 5 μ L of either ³H-Alanine, ³H-Proline or ³H-Lucine was deposited. The deposit was carefully dried with a visible light curing dental unit. Each specimen was placed in a 50 mL conical flask containing 20 mL artificial saliva. The flasks were sealed with rubber stoppers and placed in a water-bath adjusted to 37°C with gentle shaking (80 times/minute).

At time intervals starting from 0.25 hrs. from immersion, and up to 30 days, 50 μ L aliquot samples were collected. The aliquot removed was replaced with equal amounts of saliva so that saliva volume remained at 20 mL. The 50 μ L aliquot samples were mixed with 10 mL Optiphase scintillation cocktail. Radioactivity of all collected samples together with their respective standard doses were counted using the liquid scintillation counter.

Means of each six samples at each time interval and standard deviations were computed. One way analysis of variance (ANOVA) was conducted to discern the differences within and among the three experimental groups.

Influence of drying method on radio-labelled amino acid

In the experimental procedure each cavity receives 5 μ L of radio-labelled amino acid and is then dried. Drying each individual cavity using a dental visible light source proved to be tedious especially when hundreds of such cavities are to be dried in future experiments. Drying under vacuum in a glass bell jar will permit drying 30 or more cavities simultaneously. The method of drying could, however, influence the adsorption of radioactivity on the cavity floor, hence, its rate of release. The selection of the drying method was determined experimentally.

The preceding experimental procedure for radiotracer selection was duplicated, except that drying of the deposited radio-labelled amino acid was achieved under 85 torr vacuum in a bell jar for one hour. Thus, two groups of 18 teeth each, one group dried by visible light and the other group dried under vacuum, were tested. A factorial analysis of variance was conducted to determine whether the drying method and ^3H -labelled amino acid interaction influences the release of the radioactivity.¹³

Seepage of ^3H -Alanine molecules through enamel and dentin

Amino acid molecules are smaller than glucose molecules. Alanine showed most promise for these experiments. Alanine has the smallest molecular size among the 3 amino acids considered. Accordingly, the concern that it may seep through enamel and dentin (given the presence of the nail enamel seal) has to be resolved experimentally. Six molar teeth were taken randomly from over 100 selected for several studies. Class V cavities were prepared and ^3H -Alanine was deposited and dried. Each cavity as well as the root apices were sealed with inlay wax. The teeth, however, were not sealed with nail enamel. Each tooth was placed in a conical flask containing 50 mL of artificial saliva at 37°C with gentle shaking. Samples (50 μ L) were taken at time intervals starting from one hour and up to 60

days. Aliquot removed was replenished. Each sample was mixed with 10 mL Optiphase scintillation cocktail. The presence of any radioactivity was counted using a scintillation counter.

Sensitivity assessment of micropipette and investigator

The volume of ^3H -Alanine to be placed in a cavity under investigation must be such that it is small enough to be accommodated by the cavity size and easy to dry. The smaller the drop of ^3H -Alanine, the less accurate is the radioactivity count, however. An experimental determination of sensitivity of micropipette and investigator's ability to control the radioactivity counts was conducted. Sodium iodide ^{125}I * was used. Micropipettes capable of measuring 0.5, 1 and 5 μ L were used. Each pipette was used to draw 9 samples of the radioactive material. The radioactivity count in each sample was determined using a Gamma Counter®.

The mean counts per minute and standard deviation was determined for each volume group.

Results and Discussion

The following data are reported after a system was elected to deal with aberrant values.^{14,15}

Scintillation cocktail with high radioactivity recovery

The radioactivity recovery results for the standard and saliva-containing samples are shown in Table 1.

Table 1. ^3H -Alanine radioactivity counts.

Dose Volume	Radioactivity "Counts/min." (Means of 6 Samples)			
	Optiphase	Optiphase + Saliva	Lumagel	Lumagel + Saliva
10/ μ L	69620	70541	51419	545627
20 μ L	155134	158011	147292	174331
30 μ L	214910	192292	245325	254814
40/ μ L	304002	260210	313132	290690
*50/ μ L	384972	348030	385874	413357
60 / μ L	449977	396130	479938	641609

Percent correlation coefficients R:

Optiphase R = 0.99751

Lumagel R = 0.98974

* Radio Chemical Center, Amersham, England.

© Beckman Model 4000, Beckman, CA, USA.

Both Optiphase and Lumagel showed high recovery values. Correlation coefficient for each cocktail when used by itself relative to when it was used with saliva was quite high. Optiphase scintillator was adopted for radioactivity measurements in the microleakage studies since its correlation coefficient was somewhat higher than that for Lumagel.

Influence of volume of saliva and aliquot sample

Table 2 shows the mean total radioactivity counts in different volumes of artificial saliva and for different aliquots. Analysis of variance results, shown below the table, suggested that the amount

Table 2. Influence of volume of saliva and volume of withdrawn samples on accuracy of measured radioactivity at various times.

Mean total radioactivity in Saliva (Counts/Min.)				
Volume of saliva, mL	Time	Volume of pipette μ l		
	T	10	50	100
10	T ₁	277000	337960	317900
	T ₂	268500	306260	276500
	T ₃	269300	302300	270530
	T ₄	266000	288800	242650
	T ₅	291300	307960	265330
20	T ₁	272600	290600	286860
	T ₂	277000	315000	269260
	T ₃	285600	280520	256300
	T ₄	266000	292520	273900
	T ₅	305600	283920	256700
50	T ₁	252500	335300	337250
	T ₂	356500	269300	325000
	T ₃	246500	274500	255900
	T ₄	210000	261300	253150
	T ₅	191500	230300	254000
100	T ₁	225000	282500	304500
	T ₂	278000	300000	306300
	T ₃	250000	332000	274800
	T ₄	223000	303000	281000
	T ₅	395000	331000	266500
Sources of Variation	df	Sum of Squares	Mean Square	
Between groups	3	3.35791 E + 09	1.119304 E + 09	
Within Groups	56	7.13187E + 09	1.273548E + 09	
Totals	59	7.46766 E + 10		

Calculated F value = 0.8788863 p > 0.1

of radioactivity was neither influenced by volume of immersion saliva, nor by volume of aliquot withdrawn at various intervals. The amount of saliva used as a medium can vary from 10-100 μ l without influencing the accuracy of radioisotope readings. The sample of aliquot withdrawn for each reading can similarly vary from 10-100 μ l without influencing the accuracy of radioisotope readings.

Influence of incubation temperature on change of saliva volume

The data in Table 3 show the means of five readings at each time interval and in each condition. The coefficient of variation (CV%) for the radioactivity was 8.57% for the sample kept at 37°C and 12.19% for the sample kept at room temperature. The student t test for the two sets of data showed no significant difference among them at all confidence levels.

Table 3. Influence of incubation temperature and shaking on concentration of radioactivity.

Time (Days)	Radioactivity (Counts/min./50 μ l)	
	At 37°C + Shaking	At room temperature no shaking
0	6844	6838
1	6452	6350
2	6373	6264
5	6958	6079
6	6270	6537
7	6982	6645
8	6577	6396
9	6602	6585
12	7427	7079
14	6907	6789
16	7012	6693
19	7188	7290
21	7434	7368
28	7216	7111
33	7823	7797
40	8538	9383
47	7739	7485
Mean	7070.375	6965.643
Stand. Dev.	606.3313	849.7406
Stand. Err.	151.5828	227.1027
CV%	8.57566	12.19903

Calculated t value = 0.3922953, p > 0.5.

The result showed that the incubation temperature of 37°C and gentle shaking had no effect on the volume of artificial saliva for a period as long as 47 days. Thus, the microleakage experiments can be carried out under these conditions with no significant effect on the volume of the saliva immersion medium, and hence on the concentration of the aliquot.

Effectiveness of nail-enamel as a sealant

All four aliquots of teeth where cavities were exposed showed radioactivity. All four aliquots of teeth completely sealed with a nail varnish showed only background levels of radioactivity. Absence of radioactivity in the specimens completely sealed off with nail enamel indicated the effective sealing ability of nail enamel.

Radioactivity retention by nail enamel

The means of each of five readings at a given time interval are reported in Table 4. The mean calculated value for the radioactivity is supposed to be 6250 counts/min/50 μ l. Since the experimental means were somewhat greater than the calculated means, one may conclude that nail enamel does not retain the released radioactivity.

Seepage of radioactive tracer into dentinal tubules

In all cases, all counts were within background level indicating that the dentin slices had no radioactive material. It is concluded that the

Table 4. Radioactivity retention by nail enamel.

Time (Days)	Mean radioactivity around sealed whole tooth (Counts/Min/50 μ l)
0	7002
1	7127
2	6808
5	6912
6	6688
7	6734
8	6623
9	6738
12	6839
14	6977
16	7182
19	6870
21	6958
28	7473
33	6952
40	6644
47	7116

radioactive material did not seep through the dentinal tubules to any measurable depth.

Selection of radiotracer

The means of six readings of radioactivity release at each given time interval are shown in Table 5. One way analysis of variance results are shown below the table. The means show that ³H-Alanine yielded the highest radioactivity release both at less than one day and at 30 days. Analysis of variance resulted in strong significant difference (99%) between the release rates of the three ³H-labelled amino acids. ³H-Alanine seems to be the radiolabeled material of choice for microleakage studies since it showed maximum release.

Influence of Drying Method

Results comparing the radioactivity release following vacuum drying as compared to its value after visible light drying are shown in Table 6.

Factorial ANOVA showed that there was a significant difference at 95% levels in the release of the three amino acids whether drying was done by vacuum or by light. The factorial analysis of variance further substantiated the finding of the preceding experiment by showing significant differences, at all levels, between the release rates of the

Table 5. Release rates of three tritium labelled amino acids.

Time	Mean release % (n = 6)		
	Alanine	Proline	Lucine
15 min.	75.8	64.46	52.8
60min.	85.8	68.73001	62.96
18hrs.	58.4	37.9	49.6
24hrs.	72.6	47.6	51.5
2 days	61.0	51.2	48.8
3 days	67.3	57.7	52.6
6 days	85.3	67.1	58.46
8 days	81.4	71.3	54.6
10 days	76.1	69.96	59.4
13 days	79.5	75	60.1
16 days	77.8	82.2	67.3
20 days	85.0	68.4	75.3
24 days	77.8	67.3	62.3
30 days	82.9	46.4	64.1

Source of Variation	df	Sum of Squares	Mean Square
Between groups	2	2397.897	1198.948
Within groups	39	3750.317	96.16196
Total	41	6148.213	

Calculated F = 12.46801, p < 0.01

Table 6. Influence of the drying method on radioactivity release rate.

Time	Alanine		Proline		Lucine	
	Light	Vacuum	Light	Vacuum	Light	Vacuum
15min.	75.8	78.7	64.5	75.1	52.8	80.4
60min.	85.8	79.5	68.7	69.7	62.9	88.0
18hrs.	58.4	57.2	37.9	52.6	49.6	72.1
24hrs.	72.6	62.2	46.4	38.4	51.5	72.7
2 days	61.0	67.2	47.6	39.8	48.8	71.4
3 days	67.3	65.3	51.2	51.2	52.6	78.3
6 days	85.3	82.3	57.7	46.3	58.4	82.3
8 days	81.4	77.5	67.1	54.8	54.6	78.2
10 days	76.1	82.6	71.3	62.6	59.4	72.9
13 days	79.5	72.4	69.9	52.3	60.1	73.8
16 days	77.8	87.7	75.0	60.1	67.3	99.6
20 days	85.0	90.2	82.2	63.9	75.3	93.5
24 days	77.8	77.1	68.4	63.2	62.3	74.1
30 days	82.9	78.4	67.3	58.9	64.1	68.6

Source of Variation	df	Sum of Squares	Mean Square
Within groups	78	7466.563	95.72516
Between groups	5		
Factor 1	1	436.5938	436.5938
Factor 2	2	3816.313	1908.156
Interaction 1x2	2	2757.313	1378.656
Total	83	14476.78	

Calculated F for Factor 1 = 4.5609

(Factor 1 compares light and vacuum drying for 3 amino acids),
p<0.05.

Calculated F for Factor 2 = 19.9337

(Factor 2 compares the three different amino acids), p < 0.01.

Calculated F for interaction 1x2 = 14.40224

(compares interaction of factor 1 and factor 2), p < 0.01.

Table 7. Influence of micropipette volume on accuracy of radioactivity counts.

Sample #	CPM per micropipette volume		
	0.5/ μ L	1.0/ μ L	5.0/ μ L
1	389757	120282	577166
2	398982	79834	628896
3	130890	148700	576903
4	423875	122389	605504
5	349356	115491	605616
6	380779	163335	597522
7	236809	143272	601764
8	294404	162672	610705
9	490458	136564	593050
	Mean CPM = 343913	Mean CPM = 132504	Mean CPM = 599680.6
	S.D. \pm = 108215	S.D. \pm = 26388.1	S.D. \pm = 16289.9
	C.V.*% = 31.46%	C.V.*% = 19.929%	C.V.*% = 2.716%

C.V. = Coefficient of Variation

three amino acids irrespective of the drying method. Also the analysis proved that differences in release rates exist at all levels when both the type of amino acid and the method of drying were considered.

Seepage of ^3H -Alanine through enamel and dentin

All samples and up to 60 days showed only background level radioactivity counts. This finding suggests that Alanine did not seep through enamel or dentin.

Sensitivity of Micropipette

The results of this experiment are shown in Table 7. The table shows that the least coefficient of variation (2.716%) was obtained when a 5 μ L pipette was used to deposit the radioactivity in a cavity. The coefficient of variation was nearly 31% and 20% when 0.5 μ L and 1.0 μ L micropipette were used, respectively. Thus, a 5 μ L radioactive material is the volume of choice to deposit in the cavity or root canal being tested.

Using artificial saliva as a diffusion medium at 37°C and gentle shaking.

In addition to 37°C and gentle shaking, closer simulation to the physico-chemical oral environmental conditions surrounding the tooth is more likely when the immersion medium is artificial saliva. The chemical composition of the artificial saliva used in this proposed research is depicted in

Table 8. Composition of VA-Oralube artificial saliva*.

KCl	2.498 gm
NaCl	3.462 gm
MgCl ₂	0.235gm
CaCl ₂	0.665 gm
K ₂ HPO ₄	3.213gm
KH ₂ PCO ₄	1.304gm
Methyl p-hydroxybenzoate	8.0gm
NaF	17.68mgm
FD&Credye#40(2%)	1.0 ml
Flavoring	16.0g
70% Sorbitol (Sorbo)	171.0gm
Na carboxymethylcellulose	40.0 gm
Water q.s. ad	4000.0 g

* All ingredients are placed into a vessel and mixed with a T-Line Model 106 Stirrer with a 3-inch propeller; mix for 8 hours; no heating or blending is required.

Table 8. Fresh batches of saliva were prepared every 3 months. Saliva was kept refrigerated in sterile containers until it was used.

Thermal Cycling

The proposed experimental design does not take into account changes in testing temperature. Change in mouth temperature is represented *in vitro* by thermal cycling. Many of the microleakage studies discussed previously¹ have used thermal cycling. The range of temperature used in thermal cycling techniques has an upper limit of 45-60°C.

The validity of thermal cycling in studies of microleakage is questioned by many investigators. Brannstrom (1984) states, "Nor can I imagine how variations of intra-oral temperature, which are usually of short duration and within a normal range, can have any significant influence on the flow of fluid around the filling".¹⁶ Problems encountered with the thermal cycling procedure include increased crazing of the enamel initiated during cavity preparation which renders microleakage reading useless.¹⁷ In a study to evaluate the effect of thermal cycling on fracture strength and on microleakage, thermal cycling caused severe reduction in fracture strength but microleakage values were the same.¹⁸ The study suggested the invalidity of thermal cycling in leakage testing. In a laboratory study, a decreased retention of composite and unfilled resin systems with thermal cycling was also reported.¹⁹

Accordingly, it is concluded that thermal cycling is an experimental procedure that does not represent the clinical situation, changes the sources of

leakage around the restoration and changes the properties of the restorative material.

Whereas, a primary goal of this proposed research was to quantitate microleakage, the introduction of thermal cycling into the experimental procedure is deemed ill-advised.

Experimentally determined research methodology

In the light of the above studies for experimental standardization and the available literature, the reverse diffusion method, as developed originally,^{9,12} but with the use of a control group serving as a reference¹ and the application of stricter controls in accordance with the foregoing results would appear to be valid in quantitating microleakage. Specifically, each experimental condition should be represented by six restored cavities/canals. The standard dose should also be based on 6 specimens and its mean radioactivity should be determined at each experimental time interval. Microleakage from a restored cavity (mean of 6) should be expressed as a percent of the leakage that occurred from the unrestored cavity (mean of 6). This value may be termed relative microleakage value. In addition, an absolute value of microleakage from a restored cavity or root canal may be expressed as a percent of the standard dose (mean of 6). The relative value is the true *in vitro* microleakage while the absolute value is the apparent microleakage.

Each tooth should be coated with 2 layers of nail varnish leaving the cavity exposed. Each cavity should receive 2 μ l of the radioisotope tracer ³H-Alanine using a 5 μ l pipette and dried under laboratory vacuum. Each tooth should be placed in a 50 mL conical flask containing 20 mL of artificial saliva. All flasks should be placed in a 37°C water-bath and shaken gently (80 times/minute). Aliquots of 50 μ l of saliva containing the leaked radiotracer are drawn at 0.25, 0.50, one, two, three and four hours, one day, then every day up to one week then, every two days up to thirty days, then every one week up to six months, and then every month up to 18 months or beyond. Saliva should be replenished at each time. Each aliquot sample is added to 5 mL of scintillation cocktail and the amount of radioactivity in the sample is determined as CPM's using a scintillation counter.

The CPM's in each sample drawn at a time X is mathematically manipulated to yield the radioac-

tivity counts that leaked from each restored cavity (C_{rx}) at time X. The mean counts at time X for six restored cavities (C_{rx}) is computed by adding the leakage of the 6 restored teeth at time X and dividing the sum by 6. Confidence interval (at 95%) is also computed for the six specimens at time X. The mean counts for each restored group at time X, C_{rx} is then divided by the mean leakage of the 6 unrestored (U) specimens serving as control (C_{ix}), where C_{ix} is the leakage resulting from each open cavity at time X. This value, C_{ix} , is the maximum possible leakage that can occur from a cavity at time X since the latter is fully open (unrestored) and free to leak. Therefore, the quantity of microleakage (M_x) that occurred at time X from restored cavities as percent of the quantity of possible leakage from unrestored cavities (control group) at the same time X can be computed as:

Naturally, for each time increment, there will be C_{rx} and C_{ix} . Should the rate of change in microleakage, as will be indicated by the slope of the curve representing leakage versus time, dictates continuity of the experiment, gathering data could virtually be continued until Time ($X + \infty$) or until $C_{rx} = C_{ix}$ i.e. radioactive material released from the restored cavities becomes equal to that released from the unrestored cavities (the controls). In the latter condition, the restoration will serve no microleakage protection role.

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